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PRINCIPAL INVESTIGATOR: Mary Lou Cutler, Ph.D.
Mari Grazia Cerrito, Ph.D.
Treas Chopp
Weihan Wang

CONTRACTING ORGANIZATION: Henry M. Jackson Foundation for the Advancement of Military Medicine
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Mary Lou Cutler, Ph.D.; Mari Grazia Cerrito, Ph.D.; Treas Chopp and Weihan Wang

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Foundation for the Advancement of Military Medicine
Rockville, Maryland 20852

E-Mail: mcutler@usuhs.mil

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Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals that include mammotrophic hormones and locally-derived growth factors. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma, inhibits mammary differentiation and apoptosis.
We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk and Akt signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNRas expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. In addition, EGF mitogenic stimulation also inhibits Stat5 binding to its DNA binding site in the β casein promotor.

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INTRODUCTION

Epidemiological studies indicate that the age at first pregnancy and lactation have an impact on later development of breast cancer. Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals which include mammotrophic hormones and locally-derived growth factors [1]. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma [2,3], inhibits mammary differentiation and apoptosis.

There are limited human models of mammary differentiation available for study at the present time. However, the HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluency and stimulation with the lactogenic hormone mix, DIP (dexamethasone, insulin, prolactin) [4,5]. Regulation of b casein expression in HC11 reflects in vivo regulation of this protein in the mammary gland [4]. Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5 a and b and nuclear translocation of the factors [6]. In HC11 cells the activation of Stat5 is not dependent on the Ras-Erk pathway [6] and, in fact, the induction of β-casein expression can be blocked by receptor tyrosine kinase signaling at the time of prolactin addition [7-10]. It is not clear which signal transduction pathways are responsible for the inhibiton of β-casein synthesis by receptor tyrosine kinase signaling. However, the inhibition of β-casein expression by treatment of HC11 cells with EGF or Cripto [CR-1], an EGF family member, occurs through a Ras- and phosphotidylinositol-3-kinase (PI-3 kinase)-dependent mechanism [11]. Determination of the signaling mechanism(s) that are responsible for inhibiting differentiation will provide critical insight into control of this process in HC11 cells. Because inhibition of differentiation in HC11 cells appears to be dependent upon Ras, and possibly its association with PI-3-kinase, these studies focus attention on the role of Ras and its effectors in the differentiation of mammary epithelial tissue. We propose that the growth factor regulated inhibition of DIP-induced differentiation of HC11 cells results from the activation of Ras effector pathways in addition to Raf-Mek-Erk. Inhibition may require activation of the Ras-PI-3-kinase pathway and/or the Ras-RasGAP-Rho pathway.

We will test our hypothesis by constructing HC11 cell lines carrying: effector mutants of Ras which activate only a subset of effector pathways, dominant-negative (DN) mutants of proteins in the Ras-PI-3-kinase and Ras-Rho pathways, and HC11 cell lines expressing elevated levels of enhancers Ras-Raf-Mek-Erk signaling pathway. These cell lines will be used to dissect the control of differentiation using a series of markers for differentiation and cell cycle changes.

A complete understanding of the regulation of the differentiation process in mammary epithelial cells will aid in understanding the cellular changes and mechanisms leading to carcinogenesis in this tissue and allow evaluation of therapeutic strategies on the differentiation process.

BODY

The majority of the work completed during this period addressed the goals in the original statement of work as opposed to the revised statement of work for this project. Hence, the results reported here primarily address the original statement of work.

Task 1. Construction of vectors and cell lines.

Construction of HC11 Tet-Off cell lines. The HC11 cell line was transfected with the pTetOff plasmid (Clontech) and the transfected cells were selected for 10 days with G418 (200-500μg/ml). Then individual colonies were picked, expanded and screened for ability to regulate a Tet-promoter. This was accomplished by transfection with a Tet-promoter luciferase construct and assay for luciferase activity with and without Doxicyclin (0-0.5-2.0μg/ml). The results of screening for HC11 cell lines that contain a Tet- regulatable TRE are shown in Table 1. Several of the transfected cell lines, Ax-TetOff and C6-TetOff, contained a TRE that could be regulated by Dox. These cell lines (HC11-Tet Off) were used to construct lines for the regulated expression of activated Ras or dominant negative Ras.

Production of Retroviral vector Stocks and infection of HC11 cells. pREV-TRE, a retroviral vector that expresses a gene of interest from Tet-responsive element (TRE), was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The 5' viral LTR controls expression of the transcript that contains φ^+ (the extended viral packaging signal), and the hygromycin resistance (Hyg^r) gene for antibiotic selection in mammalian cells. pRevTRE also includes the *E. Coli* Amp^r gene for antibiotic selection in bacteria. The internal TRE contains seven direct repeats of the 42-bp tetO operator sequence, upstream of a minimal CMV promoter. This promotor was used to inducibly express the genes of interest in response to varying concentrations of Doxicyclin (Dox). TtA binds to the Tet-response element (TRE) and activates transcription from the minimal promoter in the absence of Dox. The plasmids pREV-TRE-RasV12 (active K-Ras 2BV12) and pREV-TRE -RasN17 (Dominant Negative K-Ras 2B(N17) were constructed by introduction of K-Ras cDNA into pREV-TRE.
Retroviral vector stocks of pRev-Tre, pRevTre-RasV12, pRevTre-RasN17 were prepared and used for retroviral infection of HC11-TetOff cells. The HC11-TetOff cell line was infected and selected in hygromycin and Doxicyclin (2μg/ml) for ten days. Six colonies were picked from Tet-Off pREV-TRE, pREV-TRE-RasV12 and pREV-TRE-RasN17 plates and seeded in 24 well plates. These cells was expanded and tested with or without Dox for the presence or absence of Ras RNA by Northern Blot. The activation of Ras transcription following the removal of Dox from the media can be seen in Figure 1. The RasV12 line in the top panel shows an increase in vector encoded Ras following removal of Dox for 24 hours. The bottom panel demonstrates the increase in RasN17 transcripts in several clones in response to the removal of Dox for up to 72 hours 72 hours. Most clones will express at least a two fold increase in V12Ras or N17 Ras in response to Dox removal. A few of the clone Example N17b, show a >5 fold increase in expression in response to the removal of Dox.

Task 2. Determination of the effect of dominant negative expression on differentiation and Stat5 activation.

Chemical inhibitors of signal transduction pathways were used to determine the contribution of signaling pathways to the ability of EGF to block HC11 lactogenic differentiation. Using HC11-luci cells we determined that the inhibition of the Mek-Erk signaling by PD98059 and phosphotidylinositol-3-kinase (PI-3-kinase) signaling by wortmannin, enhanced differentiation as measured by activation of the beta-casein promotor driven luciferase expression. Moreover, these compounds (PD98059 and wortmannin) prevented the inhibition of HC11-luci cell differentiation by EGF. (Figure 2). The results of this study are in contrast to an earlier study that indicated Erk activation did not contribute to EGF signaling in HC11 cells [11].

Exposure to the mitogen EGF regulated expression of cyclin D and Mek1 in HC11 cells. HC11-luci cells were grown to confluence then exposed to DIP or DIP plus EGF (10 ng/ml) for the indicated period of time. The presence of specific signal transduction proteins was detected by Western blotting. Blots were reacted sequentially with antibodies for Ras, Rac and RhoA and Mek1, Erk 1, cyclin D and p27 ^{KIP}. Cyclin D and Mek1 exhibit decreased expression during differentiation but the addition of EGF to cultures restores the expression (Figure 3). The other protein levels were less affected. These results suggested that EGF stimulation is sufficient to prevent growth arrest normally detected prior to induction of differentiation. EGF stimulation may function by preventing the down-regulation of Mek1 expression, thereby contributing to the stimulation of Erk activation.

EGF stimulation of HC11 cells results in activation of Ras. EGF stimulation of HC11 cells also results in activation of Ras (i.e. conversion to the GTP-bound state) but not Rho. Using activity assays for activated Ras and activated Rho, the % activated Ras or Rho was determined following serum starvation and stimulation of HC11 cells with EGF. The results are shown in Figure 4 and indicate that 12% of the Ras protein is converted to the GTP-bound state following EGF stimulation. The RasGTPase is a central activator of signal transduction pathways including Mek-Erk and Akt. Hence, Ras activation by EGF stimulation of HC11 cells contributes to the activation of Ras effectors. However, in this cell background the activity of another small G protein, the RhoGTPase, is not readily stimulated (or the stimulation is too fleeting to detect in this assay).

EGF elevates AKT phosphorylation and Rho kinase activity. The effect of EGF stimulation on signal transduction pathways was assayed in HC11 cells. HC11-luci cells (carrying a β casein-luciferase reporter construct) were used in this experiment. Cells were grown to confluence and induced to differentiate with DIP. Cell lysates were collected at specific times post induction and the activity of Erk, Rho kinase and Akt kinase activation were determined. In the case of Erk and Rho kinase immune kinase activity assays were used. Akt activation was detected using an antibody that detects the phosphorylated form of the protein (phospho serine 473). The results are shown in Figure 5 and indicate that EGF stimulation results in a transient activation of Erk. The result of mitogenic stimulation of HC11 cell by EGF is more sustained Rho kinase and Akt activation. These results correlate well with the signal transduction inhibitor studies. Those experiments determined that inhibition of Erk and Akt pathways signaling could protect cells from EGF disruption of differentiation. However, the stimulation of Rho kinase activity is somewhat puzzling because the Rho GTP loading was not detected in response to EGF.

Dominant negative Ras (N17Ras) adenovirus has been used to infect HC11 cells. As a proof of principle experiment a dominant negative Ras (N17Ras) adenovirus has been used to infect HC11 cells to evaluate the ability of adenovirus to infect the HC11 cells and to determine the effect of blocking Ras-based signal transduction on lactogenic differentiation. As illustrated in figure 5 infection of HC11-luci cells resulted in significant DNRas expression. The expression of DNRas protein was determined by western blotting following infection with various M.O.I. of DNRas adenovirus. The lysates of infected cells were isolated following 24 hours of infection without the induction of differentiation or 48 hours of infection which included 24 hours of DIP induction for lactogenic differentiation. The results demonstrate that the DIP induction of lactogenic differentiation does not inhibit adenovirus expression of DNRas protein.

To determine the effect of DNRas on DIP-induced HC11 lactogenic differentiation and its inhibition by EGF purified adenoviral vector stocks of control (empty vector) and DNRas adenovirus were used to infect HC11-luci cells at a M.O.I of 10 (see above). Cells were infected for 24 hours in growth media followed by incubation for 24 hours in DIP-induction media in the presence or absence of EGF. The cells were lysed and β-casein promotor regulated luciferase activity was determined. As illustrated in Table 2 DNRas infected cells exhibited activation of the β-casein promoter even in the absence of DIP and there was no inhibition of promotor activity when induction was performed in the presence of EGF (10ng/ml). This was in contrast to the vector control cells in which β-casein promotor regulated transcription was completely inhibited by EGF. The level of activation in the absence of dexamethasone or prolactin was surprising, but because the induction was performed in the presence of complete serum-containing media low levels of glucocortocoids and prolactin in serum may have contributed to the promotor activation. Inhibition of ras-dependent signal transduction by DNRas enhanced β-casein promotor activity and prevented inhibition by EGF. This result confirms previous studies using farnesyl transferase inhibitors and demonstrates that Ras-dependent signal transduction negatively regulates lactogenic differentiation in HC11 cells. The results also suggest that signal transduction from the Ras pathway may function to repress transcription of β-casein. Moreover, these studies indicate that adenoviral vector expression can be used in HC11 cells to probe the signal transduction pathways during this process.

EGF inhibits Stat5 binding to its binding site in the β casein promotor sequence.
The ability of Stat5 to bind to the Stat5 DNA binding site in the β casein promotor following induction of lactogenic differentiation in the presence and absence of EGF was assayed by EMSA. HC11 mammary epithelial cells were grown to confluency in the presence of 10 ng/ml EGF and then maintained for 3 days without EGF. The cells were starved for 24 h in serum-free media prior to induction for 15 min and 48 h with the lactogenic hormones (10^{-6} M dexamethasone, 5 μg /ml insulin, and 5 μg /ml ovine prolactin). Nuclear extracts were prepared and EMSAs were performed by incubating 10 μg of nuclear protein with the Stat5 DNA binding site from the beta-casein [12]. Specific binding was analyzed on a 6% DNA retardation gel. For supershift assays, nuclear extracts were preincubated with antibody for 15 min prior to the addition of the labeled probe. The results shown in figure 7 indicate that EGF at either 10 ng/ml or 100 ng/ml inhibited Stat5 DNA binding following 15 minutes of lactogenic hormone stimulation of the HC11 cells. Stat5 binding was difficult to detect at 48 hours of differentiation.

Activated V12Ras expression blocks β casein transcription in Tet regulatable HC11 transfectant cell line. HC11 transfectant cell lines expressing activated V12Ras or dominant negative N17Ras were used to test the ability of Ras expression to alter lactogenic differentiation. Vector control, TRE-v12Ras and TRE-N17Ras cell lines were grown to confluence in Dox containing media then incubated without Dox for 48 hours prior to the induction of differentiation with DIP. RNA was harvested from cells at 0, 48,72 and 96 hours post addition of DIP and used to determine the level of Ras and β casein expression by Northern blotting. The results in figure 8 indicate that the 5 fold increase in v12Ras expression inhibited β casein expression by 3 fold compared to the control cell line. The expression of dominant negative N17Ras did not affect the level of β casein induction compared to the control. This result suggests that the Tet regulatable system established by us will be a useful tool for testing the ability of signal transduction pathways to alter lactogenic differentiation in HC11 cells.

KEY RESEARCH ACCOMPLISHMENTS

-Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.

-Construction of HC11 cell lines expressing RasV12 and RasN17 under the control of a regulatable promotor.

-Demonstartion that EGF disrupts differentiation via stimulation of the Erk and Akt pathways.

-Demonstration that EGF stimulation results in accumulation of GTP-bound Ras.

-Demonstration that DNRas adenovirus can be used to infect HC11 cells and that DNRas expression enhances activation of the β casein promotor.

REPORTABLE OUTCOMES

- Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.

CONCLUSIONS

We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk, Akt and other signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNRas expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. In addition, EGF mitogenic stimulation also inhibits Stat5 binding to its DNA binding site in the β casein promotor.

This data focuses on the role of two Ras effector signal transduction pathways (Erk and Akt) in preventing mammary epithelial cell differentiation. Our results indicate that inhibition of either or both of the pathways should prevent the disruption of differentiation by mitogens of the EGF family. This approach to regulating differentiation may be useful in designing therapeutic approaches using signal transduction inhibitors (STIs).

REFERENCES

1.Snedeker, S, C Brown, and R Diaugustine. 1992. Expression and functional properties of TGFa and EGF during mouse mammary ductal morphogenisis. Proceedings of the National Academy of Sciences **88**, p276-280.

2.Dotzlaw, H, T Miller, J Karvelas, and L Murphy. 1990. Epidermal growth factor gene expression in human breast biopsy samples: relationship to estrogen and progesterone receptor gene expression. Cancer Research **50**, p4204-4208.

3. Mizukami Y, A nomura, M Noguchi, T Taniya et al. 1991. Immunohistochemical study of oncogene product Ras p21, c-myc, and growth factor EGF in breast carcinomas. Anticancer research 11, p1485-1494.

4. Danielson, K., C. Oborn, E. Durbam, J. Butel, and D. Medina. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proceedings of the National Academy of Sciences. **81**, p. 3756-3760.

5. Ball, R., R. Friis, C. Schonenberger, W. Doppler, and B. Groner. 1988. Prolactin regulation of a b-caseingene expression and a cytosolic 120kD protein in a cloned mouse mammary epithelial cell line. EMBO Journal. **7**, p. 2089-2095.

6. Wartmann, M., N. Cella, P. Hofer, B. Groner, X. Liu, L. Henninghousen, and N. Hynes. 1996. Lactogenic hormone activation of Stat5 and transcription of the b-casein gene in mammary epithelial cells is independent of p42Erk mitogen-activated protein kinase activity. Journal of Biological Chemistry. **271**, p. 31863-31868.

7. Hynes, N., D. Taverna, I. Harwerth, F. Ciardiello, D. Salomon, T. Yamamoto, and B. Groner. 1990. Epidermal growth factor, but not c-erb-2, activation prevents lactogenic hormaone induction of b-casein gene in mouse mammary epithelial cell line. Molecular and Cellular Biology. **10**, p. 4027-4034.

8. Marte, B., M. Jeschke, D. Grause-Porta, D. Taverna, P. Hofer, B. Groner, Y. Yarden, and N. Hynes. 1995. Neu differentiaiton factor/heregulin modulates growth and differentitaion of HC11 mammary epithelial cells. Molecular Endocrinology. **9**, p. 14-23.

9. Merlo, G., D. Grause-Porta, N. Cella, B. Marte, D. Taverna, and N. Hynes. 1996. Growth, differentiation and survival of HC11 mammary epithelial cells: diverse effects of receptor tyrosine kinase-activating peptide growth factors. European Journal of Cell Biology. **70**, p. 97-105.

10. Peterson, H. and L. Haldosen. 1998. EGF modulates expression of Stat5 in mammary epithelial cells. Experimental Cell Research. **243**, p. 347-358.

11. DeSantis, M., S. Kannan, G. Smith, M. Seno, C. Bianca, N. Kim, I. Martinez-Lacaci, B. Wallace-Jones, and D. Salomon. 1997. Cripto-1 inhibits b-casein expression in mammary epithelial cells through a p21Ras- and Phosphatidylinositol 3'-kinase-dependent pathway. Cell Growth and Differentiation. **8**, p. 1257-1266.

12. Olayioye, MA, Ibeuvink, K Horsch, JM Daly, NE Hynes. 1999. ErbB receptor-induced activation of Stat transcription factors is mediated by src tyrosine kinase. J Biol. Chem **274**, p17209-17218.

APPENDIX

The figures cited in the body of the report and the figure legends are contained in the attached Appendix.

TABLE 1

CELL LINE[a]	+DOX[b]	-DOX[b]
Ax-TetOff	1.0	11.8
C6-TetOff	1.0	18.0
D6-TetOff	1.0	2.1
A6b-TetOff	1.0	1.6
A2a-TetOff	1.0	4.8
D6b-TetOff	1.0	1.8
A6bx-TetOff	1.0	1.6

a - HC11cell line pTetOff plasmid transfectants.

b - HC11-TetOff transfectants were screened for ability to regulate a Tet-promoter luciferase activity with and without Doxicyclin. Values are fold increase in luciferase activity over cells in presence of Dox.

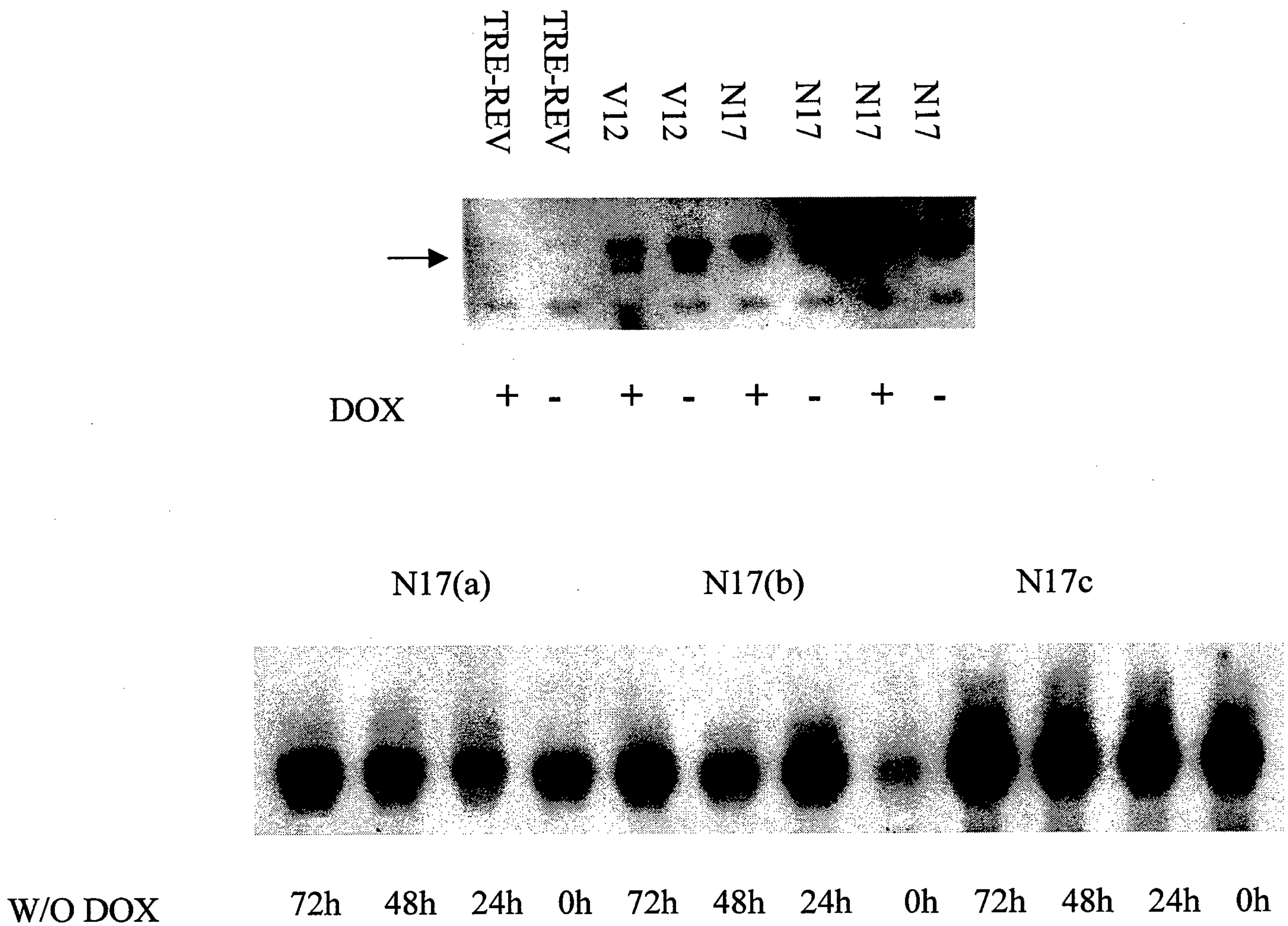


Figure 1 : Activation of Ras transcription following the removal of DOX from the media

Figure 2. HC11cells which contain a plasmid containing a luciferase gene under the control of a β-casein promotor (HC11 luci cell line) were used to test the effect of signal transduction inhibitors in EGF disruption of differentiation. HC11 luci cells were induced to differentiate in DIP-induction media with and without EGF (10ng/ml). Inhibitors were added at the time of induction. Cell lysates were harvested 48 hours after transfer to DIP-induction media and luciferase activity in lysates was determined. Results are presented as fold induction from the mean of six determinations. Inhibitors were added at optimal concentrations (PD98059-20uM, LY294002-10uM, wortmannin-100nM, SB203580-10uM and tyrphostin A25-100uM) determined via dose response curve (data not shown). Luciferase activity was normalized to cell protein.

First, we investigated the effects of the signal transduction inhibitors on DIP-induced cells in the presence of EGF.
A. DIP-induction media containing serum with and without EGF (10ng/ml).
B. DIP-induction media containing no serum with and without EGF (10ng/ml).
*These results represent statistical significance (p-value .001) over EGF alone. Both the Mek1 inhibitor (PD98059) and the PI3 kinase inhibitors (LY294002 and wortmannin) rescued the cells from EGF disruption thereby allowing the cells to differentiate.

Second, we investigated the effects of the signal transduction inhibitors on DIP-induced cells in the absence of EGF.
C. DIP-induction media containing serum without EGF. PD98059 and LY294002 enhanced differentiation (p-value .001) over DIP alone.
D. DIP-induction media containing no serum without EGF. PD98059 (p-value .01), LY294002 (p-value .05) and wortmannin (p-value .001) enhanced differentiation over DIP alone.
Serum and serum-free conditions were examined to make sure that PGDF had no role in differentiation disruption.

Figure 2

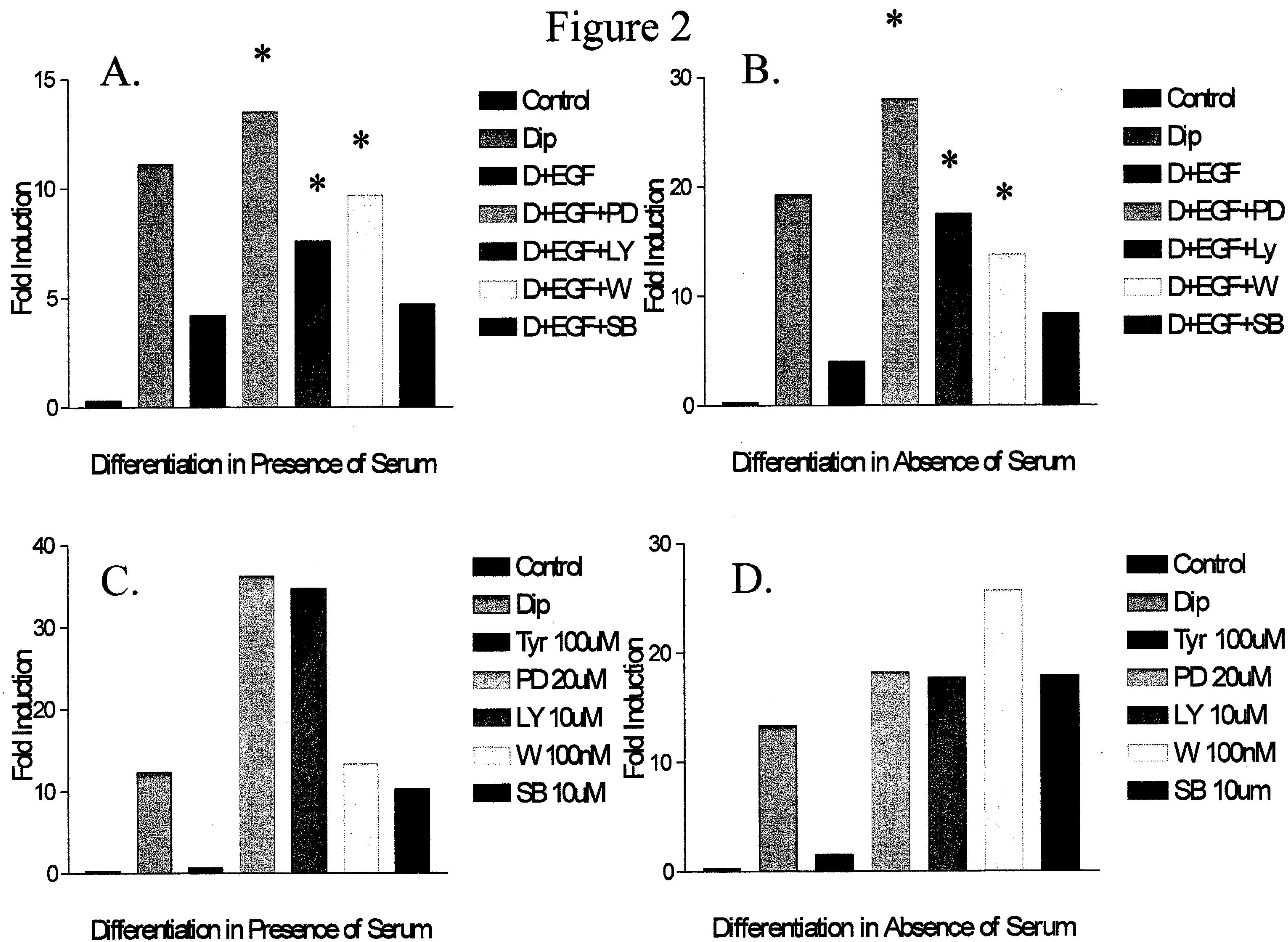
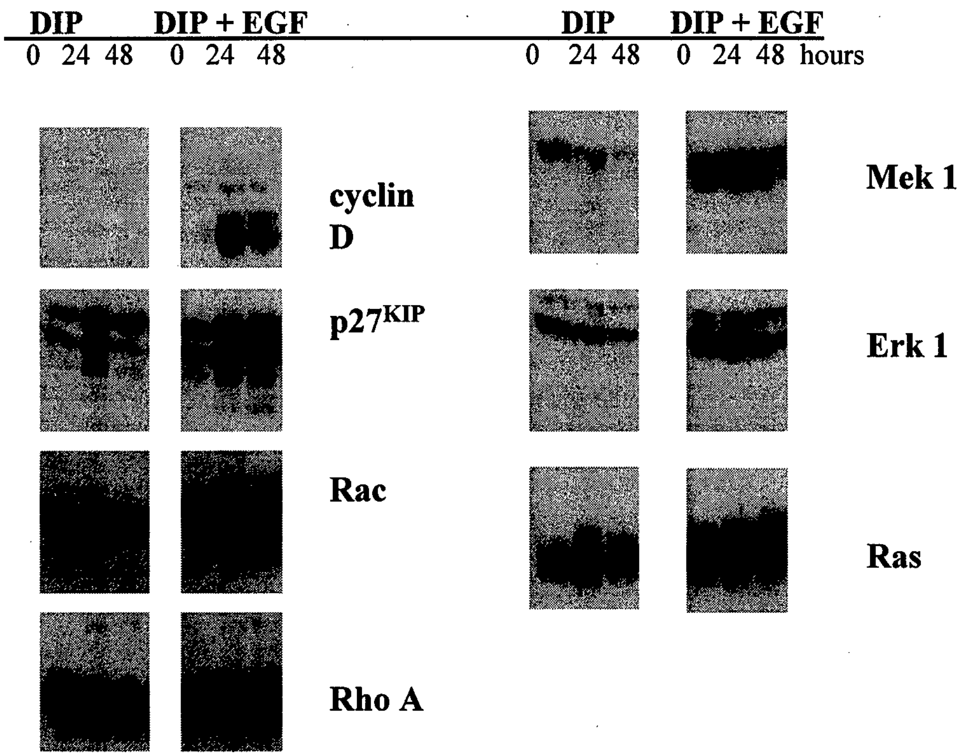

A.
15
10
5
0
Fold Induction
*
*
*
Control
Dip
D+EGF
D+EGF+PD
D+EGF+LY
D+EGF+W
D+EGF+SB
Differentiation in Presence of Serum
B.
30
20
10
0
Fold Induction
*
*
*
Control
Dip
D+EGF
D+EGF+PD
D+EGF+Ly
D+EGF+W
D+EGF+SB
Differentiation in Absence of Serum
C.
40
30
20
10
0
Fold Induction
Control
Dip
Tyr 100uM
PD 20uM
LY 10uM
W 100nM
SB 10uM
Differentiation in Presence of Serum
D.
30
20
10
0
Fold Induction
Control
Dip
Tyr 100uM
PD 20uM
LY 10uM
W 100nM
SB 10um
Differentiation in Absence of Serum

Figure 3. EGF Restores Expression of Cyclin D and Mek 1 in HC11



HC11-luci cells were grown to confluence then exposed to DIP or DIP plus EGF (10 ng/ml) for the indicated period of time. Specific signal transduction proteins in 100 μg of lysates were detected by Western blotting. Blots were reacted with the antibodies sequentially. (Ras, Rac and RhoA were detected on the same 12% gel and Mek1, Erk 1, cyclin D and p27 KIP were detected on the same 10% gel.) Cyclin D and and Mek1 exhibit decreased expression during differentiation but the addition of EGF to cultures restores the expression.

Figure 4. EGF-Activation of Ras, but not Rho, is detected in HC11 Cells

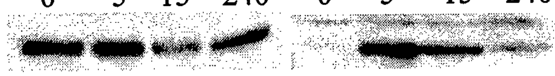
Total Rho GST-RhoBD bound

0 5 15 60 0 5 15 60 minutes DIP plus EGF

A. Rho binding

Total Ras GST-RasBD bound

0 5 15 240 0 5 15 240 minutes DIP plus EGF

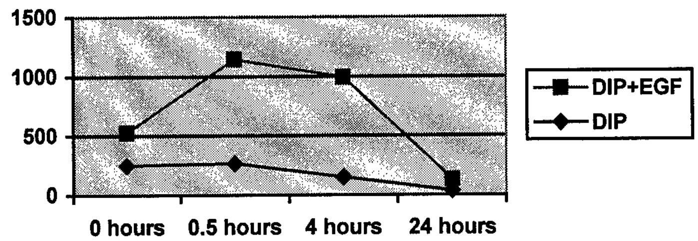


B. Ras binding

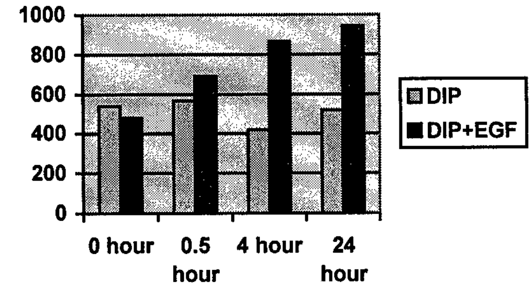
<0.1 12.2 7.3 1.2 % GTP-Ras

HC11 cells were grown to confluence in the presence of EGF (10ng/ml) then transferred to serum-free media without growth factor for 16 hours. Cells were stimulated with DIP media containing EGF (10ng/ml) for the time indicated. The cells were lysed and the lysates were incubated with the bacterially expressed glutathione transferase fusion proteins GST-RhoBD or GST-RasBD bound to glutathione beads using published methods. The amounts of Ras and Rho in the bound protein samples and in a sample of the lysate (10% of the total) were determined by Western blotting and ECL using antibodies that detect total Ras and Rho. The %Ras bound to GST-RasBD (%GTP-Ras) was calculated by densitometric scanning of the films.

Figure 5. EGF elevates Rho kinase activity and AKT phosphorylation

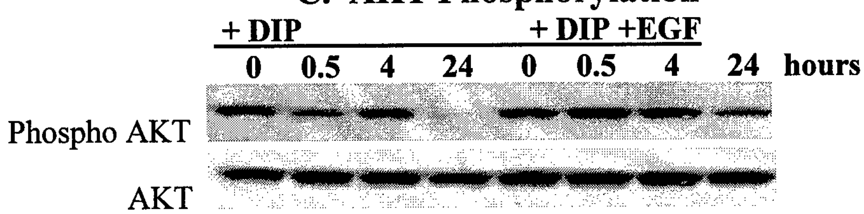


A. ERK Activity



B. RHO kinase (ROCK)

C. AKT Phosphorylation



HC11-luci cells were grown to confluence in serum and EGF (10ng/ml) containing media then changed to DIP or DIP plus EGF (10ng/ml) induction media. Lysates were harvested at 0, 0.5. 4 or 24 hours post induction and used for analysis of kinase activity.

A. Erk activation was determined following stimulation of cells with DIP or DIP plus EGF. 200 μg of lysates were used to determine Erk activity in an immune kinase reaction. Results are in cpm x 10^3. Erk activation by EGF was transient in HC11 cell.

B. Rho alpha kinase activity was determined from 500 μg of lysate in an immune kinase assay using MBP as a substrate. Rho kinase was elevated in response to EGF.

C. Phosphorylation of AKT was determined by western blotting of 100 μg of lysate with an antibody specific for phosphorylated AKT followed by reaction of the same blot with an antibody which recognizes both phosphorylated and nonphosphorylated forms of the protein.

Figure 6. Dominant negative Ras (N17Ras) adenovirus has been used to infect HC11 cells

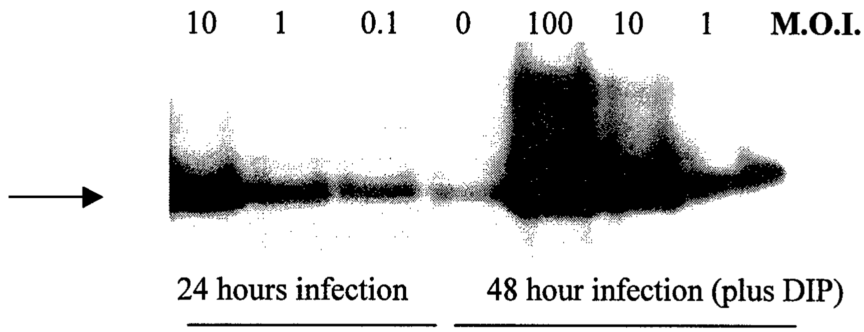


Figure 6. 5 x 10^5 HC11-luci cells were infected with 0 - 100 M.O.I. of DNRas adenovirus or empty vector in 0.2 ml. Following the 1 hour infection of cells media was added and the cells were incubated for 24 hours. At that time cell lysates were harvested (24 hour infection) or cells were exposed to DIP induction media for an additional 24 hours (48 hour infection). Cell lysates were collected after DIP induction and Ras p21 was assayed by western blotting using OP22 pan Ras mouse monoclonal antibody and enhanced chemiluminescence. The arrow indicates the position of Ras p21.

Table 2. DNRas enhances DIP-induced HC11 lactogenic differentiation

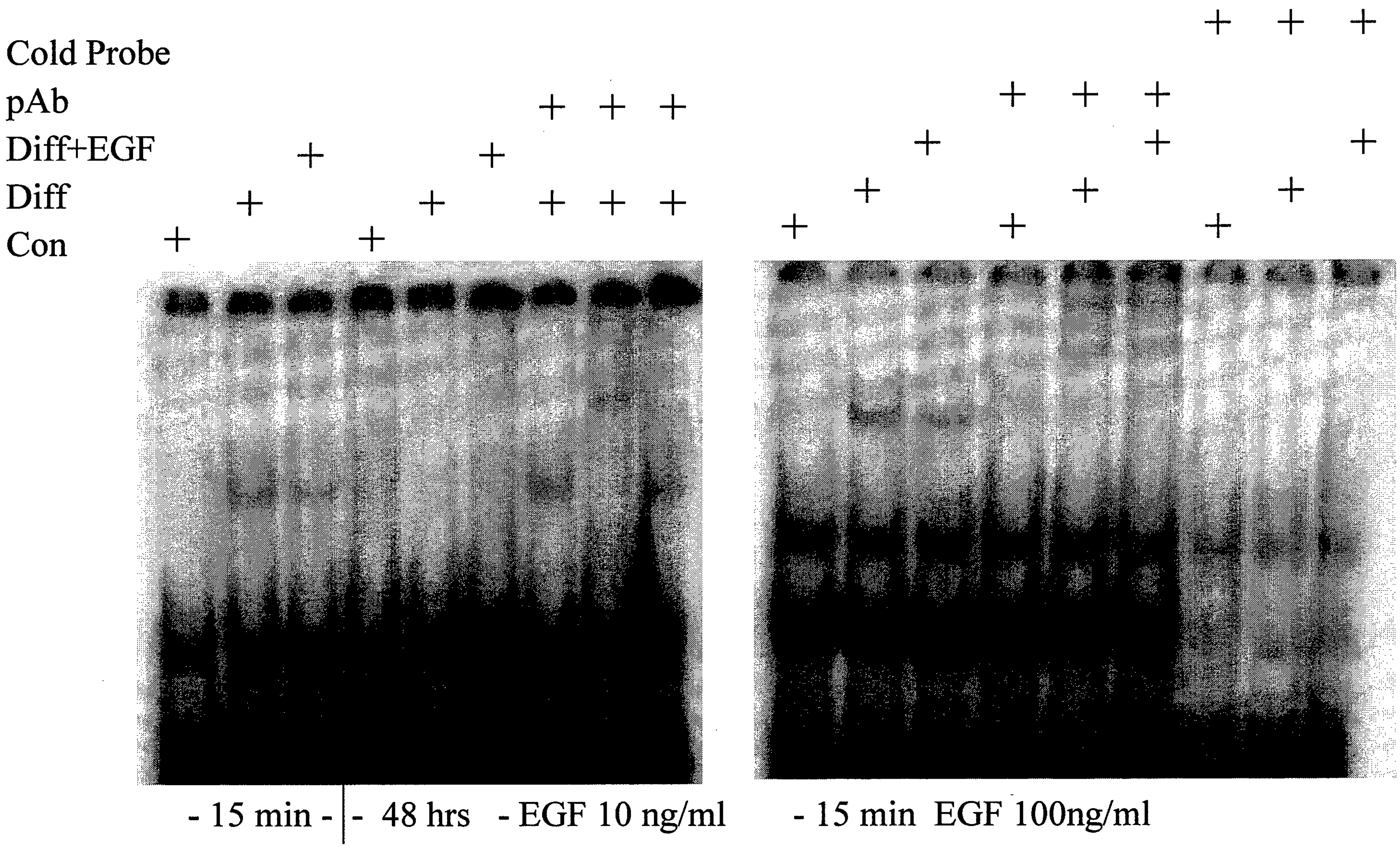
Infection	Induction		luciferase activity*
Vector	uninduced	w/o EGF	16
Vector	induced	w/o EGF	199
Vector	uninduced	+ EGF	18
Vector	induced	+ EGF	19
DNRas	uninduced	w/o EGF	359
DNRas	induced	w/o EGF	578
DNRas	uninduced	+ EGF	346
DNRas	induced	+ EGF	603

*** in relative units normalized to total cellular protein**

Table 2. Enhancement of β-casein promotor-regulated luciferase expression following infection with DNRas adenovirus. HC11-luci cells were infected with empty vector or DNRas adenovirus as described above. Following infection for 24 hours cells were incubated for an additional 24 hours in growth media (uninduced) or DIP media (induced) in the presence or absence of EGF (10ng/ml). Cell lysates were harvested and luciferase activity was measured in duplicate. Results are average of two determinations.

Figure 7. EMSA. HC11 mammary epithelial cells were grown to confluency in RPMI 1640 media containing 10% fetal bovine serum, 10 ng/ml EGF, and 5 µg/ml insulin, and maintained for 3 days without EGF. The cells were starved for 24 h in serum-free media prior to induction for 15 min and 48 h with the lactogenic hormones (10^{-6} M dexamethasone, 5 µg /ml insulin, and 5 µg /ml ovine prolactin). The EMSA method used was modification of Olayioye et al, 1999 [12]. Nuclear extracts were prepared by re-suspend cells in CEB (10 mM KCl, 20 mM Hepes, pH 7.0, 1 mM MgC12, 0.1% Triton X-100, 20% glycerol, 0.1 mM EGTA, 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 50 µM -glycerophosphate, 50 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin) and sheared using a Dounce homogenizer. Nuclei were pelleted by centrifugation and then extracted with NEB (CEB + 300 mM NaCl) by incubating for 30 min on ice. Extracts were clarified by centrifugation for 5 min at 16,000 × g. EMSAs were performed by incubating 10 µg of nuclear protein with the Stat5 DNA binding site from the beta-casein promoter (5'-AGATTTCTAGGAATTCAATCC-3') for 30 min on ice in 16 µl of EMSA buffer (10 mM Hepes, pH 7.6, 2 mM NaH2PO4, 0.25 mM EDTA, 1 mM dithiothreitol, 5 mM MgC12, 80 mM KCl, 2% glycerol, and 100 µg/ml poly(dI-dC)). Specific binding was analyzed on a 6% DNA retardation gel. The samples were loaded and electrophoresed; the gels were dried and autoradiographed. For supershift assays, nuclear extracts were preincubated with antibody for 15 min prior to the addition of the labeled probe.

Figure 7. Binding of Stat5 to DNA binding domain of β casein promotor in HC11 cells differentiated with or without EGF for 15 min or 48 hr.



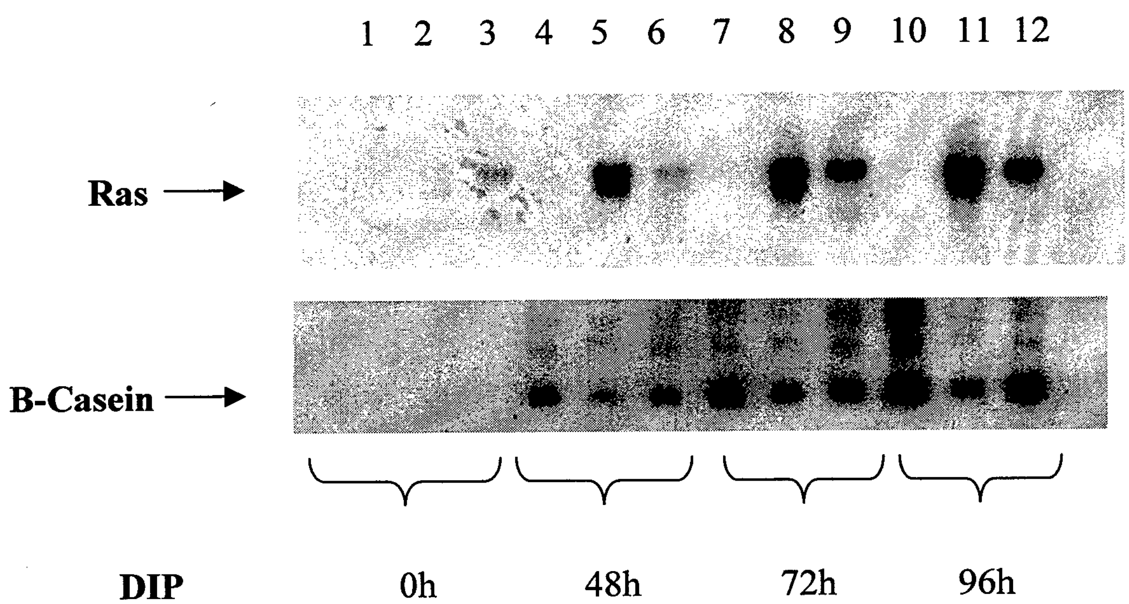


Figure 8: HC11 cells expressing activated Ras (V12Ras) and DNRas (N17Ras) were utilized to evaluate the effect of Ras-based signal transduction on lactogenic differentiation (DIP). Lanes 1,4,7,10 TRE-REV; lanes 2,5,8,11 V12; lanes 3,6,9,12 N17.